SYNTHETIC POLYPEPTIDE FOR DIAGNOSING AND TREATING PRION-RELATED DISEASES

The present invention relates to synthetic polypeptides and applies in particular to the diagnosis, prevention and therapy of several communicable, degenerative, neurological illnesses. Such illnesses are generically called spongiform encephalopathies, also prion illnesses. They occur in different mammals, for instance in the form of scrapie in sheep, BSE in cows and kuru or Jakob-Creutzfeld disease in humans.

The only molecule associated with the infecting agent found so far is a disease-specific prion protein (PrP^{Sc}) which is an anomalous isoform of a normal host protein (PrP^{C}) of unknown function. Both isoforms PrP^{Sc} and PrP^{C} coincide with respect to molecular weight and amino-acid sequence. They differ in the spatial folding and by their properties. Illustratively whereas PrP^{C} predominantly comprises α -helical secondary structures, and is soluble and protease-digestible, PrP^{Sc} comprises foremost β-sheet structures, and is insoluble and can be degraded only partly by proteases. Many indices, in particular the absence of other molecules except PrP^{Sc} , in the prion, and foremost the absence of nucleic acids, indicate that PrP^{C} assumes a significant (if not the main) role in initiating the above diseases. It is assumed that PrP^{C} proteins are able to convert normal PrP^{C} proteins into the disease-specific folding , thus explaining the infectiousness of PrP^{Sc} proteins.

Accordingly it appears very promising to develop therapies and diagnoses based on PrP^{Sc} as the central disease molecule.

Accordingly the objective of the invention is to create synthetic polypeptides offering the immunogenic properties, or in general the binding properties of PrP^{Sc} though free of its infectiousness.

This problem is solved by synthetic polypeptides defined in claim 1.

These are polypeptides containing one or more defined PrP sequences, where PrP denotes the prion protein generally independently of its conformation, these sequences being recognized by PrP^{Sc}-binding substances for instance in the mapping experiments which are described further below. There are a large number of different specifically PrP^{Sc}-binding substances. Examples are cited further below.

In summary the synthetic polypeptides of the invention therefore include at least one sequence which, in the native PrP^{Sc} is affixed to its surface where, alone or in combination with further sequences applicable within the scope of the invention, it shall form a binding site. At least one of the two β -sheet structures, or both, present in the structural model of the recombinant PrP, participate(s) in the formation of said PrP^{Sc}-specific surface structures. It is assumed that these structures act as a nucleation site in the PrP^{Sc} in the surface formation.

Synthetic polypeptides simulating binding sites present in the native PrP^{Sc} may be significant both in the therapy or diagnosis as well as regards prevention and other applications.

The invention in particular includes synthetic polypeptides comprising one or several of the following sequence segments stated in claim 2:

(a) Gly-R_1-Asp-R_2-Glu-Asp-Arg-(Tyr-Tyr)

(b) (Gln)-(Val)-Tyr-Tyr-R_3 -Pro-R_4-Asp-R_5 -Tyr-R_6-(Asn-Gln)

(c) Cys-R_7 -Thr-Gln-Tyr-R_8 -R_9-Glu-Ser-R_{10}-Ala-(R_{11} - Tyr)

(d) (Tyr-Arg)-Glu-Asn-Met-R_{12}-Arg-Tyr-Pro-Asn-(Gln-Val-Tyr)

where R_1= Asn or Ser, R_2= Trp or Tyr, R_3 = Arg or Lys, R_4 = Met, Val or Ala, R_5 = Gln, Glu or Arg, R_6 = Ser or Asn, R_7 = Val, Thr or Ile, R_8 = Gln or Glu, R_9 = Lys, Arg or Gln, R_{10} = Gln

or Glu, R_{11} = Tyr, Ser or Ala and R_{12} = His, Tyr or Asn, and where the amino acids in parentheses are not mandatorily present.

According to claim 3, further synthetic polypeptides used within the invention may contain one or more of the following sequences:

(e) Gly-Trp-Gly-Gln-Pro-His-Gly-Gly-Gly-Trp-Gly-Gln-Pro-His-Gly

(f) Lys-Pro-R_{14}-Lys-Pro-Lys-Thr-R_{14}-R_{15}-Lys-His-R_{16}-Ala-Gly

(g) Tyr-R_{16}-Leu-Gly-Ser

(h) Ser-Ala-Met-Ser-Arg-Pro-R_{17}-R_{17}-His-Phe-Gly-R_{14}-Asp

(i) Asn-Met-R_{18}-Arg-Tyr-(Pro-R_{14})-(Gln-Val-Tyr-Tyr-R_{19})

where R_{14} = Asn or Ser, R_{15} = Met, Leu or Phe, R_{16}= Met or Val, R_{17} = Ile, Leu or Met, R_{18} = His, Tyr or Asn and R_{19} = Lys or Arg and where the amino acids or sequence zones in parentheses are not mandatorily present.

The sequences of claims 2 and 3 were found in so-called mapping experiments on an immobilized peptide bank. As regards the peptide bank used (available from Jerini Biotools, Berlin), 104 peptides with 13 amino acids each are affixed by their C-terminal ends to a cellulose membrane. They cover the full sequence of PrP (hereafter PrP denotes generally the prion protein's basic amino-acid sequence regardless of conformation) and are configured in such a way as to be shifted each time by two amino acids, that is, each time 11 amino acids overlap between two adjacent peptide banks. In the course of several mapping experiments, peptide banks were loaded with different substances binding PrP^{Sc}, and the binding of these substances to special sequence zones was made visible using for instance a chemiluminescence kit (ECL, Amersham, USA).

In order to determine the sequences claimed in claim 2, a PrP^{Sc} -specific antigen denoted by 15B3 and (as found in our own pretesting, an also PrP^{Sc} -specific) recombinant bovine PrP (rbPrP) with the sequence shown in Fig. 4 were used as the PrP^{Sc} binding substances. To prepare rbPrP, illustratively a cell line (for instance E. coli) may be cultured with a vector expressing rbPrP in a suitable medium (for instance Luria broth) and then the prion protein may be isolated after being lysed from the cell inclusion bodies by further conventional purification procedures (see Hornemann et al, FEBS Letters 97, 413 (2; 277-281)).

15B3 is a monoclonal PrP^{Sc} antibody recently discovered by the inventors. Hybridoma cells producing the said (PrP^{Sc} - specific) antibodies 15B3 were filed on 13 February 1997 as DSM ACC2298 at the German Collection of Microorganisms and Cell Cultures GmbH in Brunswick.

In both cases, the two differently binding substances, that is, the antibody 15B3 and the recombinant rbPrP, recognized as the same the sequences a-d of claim 1, as reproduced for 15B3 in Fig. 1 and for rbPrP in Fig. 2.

The numerals shown in Figs. 1 and 2 denote the different bank peptide sequences bound by the monoclonal antibody 15B3 and rbPrP. The sequences of the invention each correspond the zones common to the particular spatially adjacent binding peptides. As already mentioned, Fig. 2 shows the result of a mapping experiment of which the conditions correspond to the experiment represented in Fig. 1. In this instance the antibody 15B3 was merely replaced with recombinant bovine rbPrP. Because technical difficulties preclude better reproduction of the binding sites of the recombinant rbPrP, they are emphasized by marks. These are binding sites coinciding with those of Fig. 1.

The sequences stated in claim 3 also were determined by mapping experiments. However in this instance the recognizing substance is not an antibody or rbPrP, but instead it is the Congo Red dye of which the specific binding relating to PrP^{Sc} has already been known for some time (Prusiner et al, Cell 35, 349-358, 1983). Fig. 3 shows the corresponding peptide bank with the dyed binding zones from which, as stated above, the sequences e-i were determined.

It is clear from Figs. 1-3 that the sequences a-d and e-i are not linearly related PrP sequences. As regards a 3-D model of a C-terminal fragment of recombinant mouse PrP, it was found that two of the sequences a-d stated in claim 2 are spatially close to each other. It may be assumed with high probability that when the conformation is altered, the other two sequences also will assume another position in such a way that probably all four sequences a-d shall be configured near one another in the PrP^{Sc} and are highly likely to form a conformational epitope.

Accordingly the claimed sequences represent sequence zones recognized individually in a peptide bank for instance by a PrP^{Sc} -specific antibody and which moreover very probably constitute, individually or severally, a surface binding site, for instance an epitope, in the native PrP^{Sc} -protein. The expression "epitope" denotes the specific antigen site on the surface of the PrP^{Sc} -protein which illustratively can be bound by the idiotype of 15B3.

As a result the invention prepares synthetic polypeptides which at a minimum contain one of the said PrP^{Sc} -binding substances in the sequences recognized in the peptide bank, as well as any additional ones.

Synthetic polypeptides comprising a PrP^{Sc} antigen zone already have been disclosed in the patent document WO 93/11153. The sequences stated therein represent comparatively

substantial segments of the PrP sequence. The precise boundary of a sequence for instance forming an epitope or participating in it, is lacking, and this feature hampers or makes impossible in particular the spatial buildup of minimal synthetic polypeptides having for instance the immunogen effect of PrP^{Sc}.

As discussed above, at a minimum, the synthetic peptides may be composed merely of one of the sequences claim 2 or one of the cited ones. However they may also be bound to further, suitable sequences which hereafter are called conformation sequences.

Theoretically the sequences for instance might be connected to each other illustratively by means of said conformation sequences and possibly by further sequences in such manner as to stimulate the presumed spatial configuration in the PrP^{Sc} protein. Ideally a protein (epitope) would be attained in this manner which would contain several neighboring binding sites as is the case for the PrP^{Sc}.

However, in one implementation of the invention, only one of the claimed sequences (sequence b) shall be so connected to a conformation sequence that a synthetic polypeptide is made that offers adequate binding for instance regarding 15B3, as confirmed by the inventors' tests. A polypeptide of such a configuration may include one of the two following sequences:

(j) (X)-(Gly)-Ala-Val-Val-Gly-Gly-Leu-Gly-Gly-Tyr-(R_{13})-Z-Tyr-Tyr-R_3-Pro-R_4-Asp-R_5 -Tyr-R_6- (Asn-Gln)-(Y)

(k) (X)-Tyr-Tyr-R_3 -Pro-R_4-Asp-R_5 -Tyr-R_6 -(Asn-Gln)-Z-(Gly)-Ala-Val-Val-Gly-Gly-Leu-Gly-Gly-Tyr-(R_{13})-(Y)

where X and Y are arbitrary amino-acid sequences, Z is a conventional spacer, for instance Gly-Gly, R_3 = Arg or Lys, R_4 = Met, Val or Ala, R_5 = Gln, Glu or Arg, R_6 = Ser or Asn and R_{13} = Met or Val and where the sequence zones in parentheses are not mandatorily present.

The j-sequence contains in its C-terminal zone the sequence b which is connected for instance by means of the spacer Gly-Gly to the adjoining N-terminal conformation sequence. The order is exactly the opposite in the k sequence. Other appropriate spacers in general are those assuring adequate flexibility between the connected peptide zones and exerting no influence on conformation.

Both preferentially used synthetic peptides were designed on the findings that β sheet structures occur in increased manner in PrP^{Sc}, practically always a conformation sequence inducing a β sheet structure being present up or down the sequence. The synthetic polypeptides of claim 6 therefore were provided as claimed in claim 6 with suitable conformation sequences in order to configure the epitope sequence in a β sheet structure specific for PrP^{Sc}.

In well known manner, depending on their size, polarity or charge, amino acids may be assigned into different groups. The amino acids within one group are said to be mutually homologous and there are five groups:

(1) small aliphatic, non-polar or only slightly polar acids: alanine, serine, threonine, and, within limits, glycine, proline

(2) polar, negatively charged acids and their amides: aspergillic acid, asparagine, glutamic acid and glutamine

(3) polar, positively charged acids: histidine, arginine, lysine

(4) large aliphatic, non-polar acids: methionine, leucine, isoleucine, valine, cysteine,

(5) large aromatic acids: phenylalanine, tyrosine, tryptophane.

In many cases it is possible to replace amino acids contained in peptide sequences by corresponding acids from the same group without thereby entailing a change in sequence properties. Therefore the invention also includes those sequences that do not correspond to the explicitly stated formulas but wherein one or more amino acids were replaced by a homologous acid.

Another justifiable assumption is that independently of their direction of formation, amino-acid sequences under given circumstances may offer similar binding properties, in particular antibody binding properties. In such a case they are called "retro-aminoacid sequences" which denote coinciding sequences formed in a C terminal or N terminal direction (for instance [N-terminal]- Glu-Ala-Val-Leu-[C-terminal], [N-terminal]-Leu-Val-Ala-Glu - [C-terminal]). If the amino acids being used are present in D chiral form opposite the L form of animals, then the epitope zones will be mirror symmetrical and shall also be recognized by a few antibodies, the isotypes of these antibodies differing in these properties. In such cases the terminology is "inverso-aminoacid-sequences. When both inverso and retro amino acids are used, there will be for instance coinciding epitope zones which can be unrestrictedly bound of the antibody which is specific to the original sequence. The advantage offered for instance by such retro-inverso sequences of amino acids is that the D amino acids are degraded more slowly by the organism because being recognized more poorly by the degrading enzymes. The same effect can also be achieved by substituting non-natural amino acids. Therefore the peptides of the invention also may be in retro- and/or inverso form and moreover they may contain non-natural amino acids, that is not produced by organisms. Non-

natural amino acids may be prepared by synthesizing for instance additional side chains or reactive groups in a manner to offer specific properties and matched to specific applications.

As already discussed above, the synthetic polypeptides of the invention may be used in particular in the treatment, prevention or also diagnosis of prion diseases.

A particular application is to use the synthetic polypeptides of the invention as vaccines. Illustratively an appropriate quantity of peptide is dissolved using Freund's complete adjuvant injecting it sub-cutaneously or intra-muscularly. At intervals of several weeks, again an immunogenic quantity of peptide is dissolved in Freund's incomplete adjuvant and is injected (boost). The objective of this vaccination is to induce an immune response, including endogenous production of antibodies able to specifically recognize PrP^{Sc} and neutralize or characterize it, whereby the body's own defense mechanism shall be able to forestall disease or slow it or stop it.

Another application consists in using the synthetic polypeptides in diagnosis and therapy. In the light of the prevailing conversion theory, it is assumed the PrP^{Sc} and/or PrP^{C} also bind to each other. This supposition is supported by further mapping experiments by the inventors showing (Fig. 2) that recombinant bovine rbPrp binds to sequence zones similar to those that the above antibody 15B3 binds with.

The said binding properties may be put to use for instance in therapy. Conceivably the polypeptides may be cerebrally applied to an ill patient and there they shall be available as a binding partner to the infectious PrP^{Sc}. In this manner the conversion rate might be sharply reduced and the progress of the disease slowed down. As regards diagnosis, any PrP^{Sc} contained in sample material might be specifically bound by means of the polypeptides of the invention and then be detected in appropriate manner.

The synthetic polypeptides of the invention are not restricted to the above cited sequences. Illustratively peptides in derivative form also are applicable. Illustratively such peptides might be bound to a carrier, or immunogen, such as diphteriatoxin or BSA to enhance the immune response. Another way to make derivatives is by linking to markers, for instance using biotine or peroxidase or enzymes or nucleic acids. Lastly, signal sequences might be used to facilitate the passage of the peptides into desired compartments. This latter application relates in particular to the blood/brain barrier which might be easier to cross when using signal sequences binding the transferrin receptor.

As already stated above, the synthetic polypeptides of the invention are applicable to the therapy, diagnosis and prophylaxis of prion diseases. In conjunction with all said applicabilities, it is essential that the polypeptides of the invention be administered to the patients per se or in combination with further substances, and, as already mentioned, derivatives may be used to enhance the directionality into specific compartments.

The polypeptides may be manufactured in arbitrary manner: either directly through conventional peptide-syntheses or also indirectly through RNA or DNA synthesis and then by conventional molecular-biological techniques. Accordingly another feature of the invention relates to a DNA molecule which is able to code one of the polypeptides of the invention. Preferably such a DNA molecule (where called for also in a longer sequence) is made available in an appropriate expression vector. The routines involved are conventional.

The invention furthermore relates to a kit to diagnose PrP^{Sc} or antigens against PrP^{Sc} and containing at least one of the polypeptides of the invention. This feature avails itself of the fact that the polypeptides can specifically bind with the PrP^{Sc} and with the antibodies pointing at it.

As already mentioned, one of the substances used to ascertain the polypeptide sequences may be recombinant bovine rbPrP. Surprisingly it was found that recombinant rbPrP is able to specifically bind to PrP^{Sc} and to recognize, at the corresponding peptide bank, the same sequences as the antibodies 15B3 (see Fig. 2).

Another feature of the invention therefore relates to the use of recombinant rbPrp corresponding to the sequence of Fig. 4. It was found that PrP^{Sc} specific antibodies are produced when administering recombinant bovine rbPrp of the indicated sequence. This effect can be exploited in particular with respect to prophylaxis or therapy in that recombinant rbPrP prepared as a vaccine is administered to a patient and thereby a corresponding immune response shall be triggered.

Obviously the implementation is not restricted to using bovine rbPrP per Fig. 4. Recombinant PrP sequences with species-specific deviations from the rbPrP sequence shown in Fig. 4 may be used just as well.

Lastly the invention relates also to a method for manufacturing PrP^{Sc}-specific antibodies. For purposes of immunization, at least one of polypeptides of the invention is administered in a dose sufficient for immunization to non-human mammals and the antibody thereby formed is then isolated in conventional manner.

Lastly the peptides of the invention also are suitable for the so-called pharmaceutical or chemical libraries whereby new active ingredients are tested or determined which shall specifically bind PrP^{Sc}.

SEQUENCE PROTOCOL

(1) General data

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(ii) Title of Invention: Synthetic polypeptide for diagnosing and treating prion-related diseases.

(iii) Number of sequences : 1

(iv) Computer readable version:
(a) data medium: floppy disk
(b) computer: IBM PC compatible
(c) operating system: PC-DOS/MS-DOS
(d) software: PatentIn Release # 1.0, version #1.30 (EPA)

(vi) Data of first application:
(a) Application # DE 19741607.1
(b) Date of application: 20 September 1997

(2) DATA RELATING TO SEQ ID # 1:

(i) SEQUENCE CHARACTERISTICS
   (a) Length: 219 amino acids
   (b) Species: Amino acid
   (c) Form of strand: single strand
   (d) Topology: Linear

(ii) MOLECULE SPECIES: protein

(iii) hypothetical: yes

(iv) antisense: no

(vi) PROVENANCE:
   (a) organism: bos taurus

(vii) GENOME POSITION:
   (c) units: 219

(xi) SEQUENCE DESCRIPTION: SEQ ID #: 1:

Met	Lys	Lys	Arg	Pro	Lys	Pro	Gly	Gly	Gly	Trp	Asn	Thr	Gly	Gly	Ser
1				5					10					15	
Arg	Tyr	Pro	Gly	Gln	Gly	Ser	Pro	Gly	Gly	Asn	Arg	Tyr	Pro	Pro	Gln
			20					25					30		
Gly	Gly	Gly	Gly	Trp	Gly	Gln	Pro	His	Gly	Gly	Gly	Trp	Gly	Gln	Pro
		35					40					45			
His	Gly	Gly	Gly	Trp	Gly	Gln	Pro	His	Gly	Gly	Gly	Trp	Gly	Gln	Pro
	50					55					60				
His	Gly	Gly	Gly	Trp	Gly	Gln	Pro	His	Gly	Gly	Gly	Gly	Trp	Gly	Gln
65					70					75					80
Gly	Gly	Thr	His	Gly	Gln	Trp	Asn	Lys	Pro	Ser	Lys	Pro	Lys	Thr	Asn
				85					90					95	
Met	Lys	His	Val	Ala	Gly	Ala	Ala	Ala	Ala	Gly	Ala	Val	Val	Gly	Gly
			100					105					110		
Leu	Gly	Gly	Tyr	Met	Leu	Gly	Ser	Ala	Met	Ser	Arg	Pro	Leu	Ile	His
		115					120					125			
Phe	Gly	Ser	Asp	Tyr	Glu	Asp	Arg	Tyr	Tyr	Arg	Glu	Asn	Met	His	Arg
	130					135					140				
Tyr	Pro	Asn	Gln	Val	Tyr	Tyr	Arg	Pro	Val	Asp	Gln	Tyr	Ser	Asn	Gln
145					150					155					160
Asn	Asn	Phe	Val	His	Asp	Cys	Val	Asn	Ile	Thr	Val	Lys	Glu	His	Thr
				165					170					175	
Val	Thr	Thr	Thr	Thr	Lys	Gly	Glu	Asn	Phe	Thr	Glu	Thr	Asp	Ile	Lys
			180					185					190		
Met	Met	Glu	Arg	Val	Val	Glu	Gln	Met	Cys	Ile	Thr	Gln	Tyr	Gln	Arg
		195					200					205			
Glu	Ser	Gln	Ala	Tyr	Tyr	Gln	Arg	Gly	Ala	Ser					
	210					215									